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GRANT NUMBER DAMD17-94-J-4348

TITLE: The Role of the RAS Tumor Suppressor, Lysyl Oxidase, in Breast Cancer Development and Progression

PRINCIPAL INVESTIGATOR: Sara Contente, Ph.D.

CONTRACTING ORGANIZATION: Henry M. Jackson Foundation for the Advancement of Military Medicine
Rockville, Maryland 20852

REPORT DATE: November 1997

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	November 1997	Final (28 Sep 94 - 27 Oct 97)

4. TITLE AND SUBTITLE
The Role of the RAS Tumor Suppressor, Lysyl Oxidase, in Breast Cancer Development and Progression

5. FUNDING NUMBERS
DAMD17-94-J-4348

6. AUTHOR(S)
Sara Contente, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Henry M. Jackson Foundation for the Advancement of Military Medicine
Rockville, Maryland 20852

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200)*

This project sought to analyze, in breast cell lines and normal and neoplastic human breast tissue, the pattern and level of expression lysyl oxidase messenger RNA. Lysyl oxidase expression acts as a suppressor of the transforming activity of the *ras* oncogene in mouse cells. The expression of normal *ras* was also determined. Reverse transcriptase-polymerase chain reaction (RT-PCR) for cellular RNA, and *in situ* RT-PCR on sections of fixed, paraffin-embedded breast tissue was employed. Human cancer cell lines were also transfected with a lysyl oxidase expression plasmid and examined for changes in cell growth and tumorigenic potential. Lysyl oxidase message expression was lost or severely decreased in human breast cancer cell lines. There was no corresponding increase in the expression of *ras* message. Both lysyl oxidase and *ras* were expressed in the ductal epithelium and acinar epithelium of normal breast. Expression was also detected in stromal fibroblasts, endothelial cells, and lymphocytes. In ductal carcinoma, lysyl oxidase expression was absent in some cases, patchy in others, and sometimes appeared unchanged. The expression of *ras* in ductal epithelium did not vary significantly between normal and cancerous tissue. A lysyl oxidase-transfected cancer cell line had a reduced growth rate and a less transformed appearance.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, lysyl oxidase	36
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Each of the genes in the *ras* family (H-, K-, and N-*ras*) encodes a highly similar, 21,000 dalton protein that binds GTP and functions as a component of a complex signal transduction pathway that governs the control of cell proliferation or differentiation. Mutations in the cellular *ras* gene have been clearly associated with several different human cancers. A point mutation that leads to an altered amino acid at position 12, 13 or 61 decreases the GTPase activity of the *ras* protein, locking it into an activated mode believed to contribute to uncontrolled cell growth. Such mutations in the K-*ras* gene predominate in adenocarcinomas of the pancreas, colon and lung; in thyroid tumors, mutations in each of the three genes have been found [1]. Mutations in the H-*ras* gene are associated with bladder carcinoma and uterine cervical cancer, while N-*ras* mutation is the major one found in myeloid and lymphoid disorders [1].

In the human breast, *ras* genes activated by mutation were detected in mammary carcinomas [2-4], but their occurrence was rare [5, 6]. Deletions of one c-H-*ras* allele were found in human mammary carcinoma [7, 8], but were again, rare. It was the overexpression of the normal cellular *ras* gene product, p21, that was found to be associated most consistently with human breast cancer [9]. This overexpression occurred for both messenger RNA and protein [6]. Immunoblot analysis of human primary breast carcinomas indicated that 71 percent of them contained levels of p21 protein higher than that seen in normal tissue [10]. The elevated expression of p21 is in itself not sufficient for cellular transformation in normal cells, and the contribution of this overexpression to breast cancer development and progression remains unexplained.

A model system was created in mouse to examine the effects of the overexpression of normal *ras* p21 by the transformation of NIH 3T3 fibroblast cells with a normal c-H-*ras* gene linked to the LTR of Harvey murine sarcoma virus [11]. The resulting cell line, RS485, had a transformed morphology and was tumorigenic when injected into athymic mice. NIH 3T3 cells, unlike primary cell cultures, can be transformed by *ras* alone. A flat revertant of the transformed cell line was obtained after long-term treatment with interferonβ [12, 13], and reversion persisted when the interferon treatment was discontinued. This persistence was regardless of the resumption of high levels of *ras* mRNA expression and p21 production, which had been depressed by interferon. A gene that was differentially expressed between the transformed and persistent revertant cell lines was isolated from a cDNA library; it was found to be expressed in NIH 3T3 and in the persistent revertant, but only marginally expressed in the transformed line. When the persistent revertant cell line was transfected with a plasmid construct that expressed an antisense message to this proposed suppressor, retransformed colonies could be isolated [14], indicating that the expression of this gene was causal, and not incidental, to the reversion. The amount of suppressor messenger RNA detected in the retransformed lines was again negligible; the transforming effect of the p21 expressed in these cells was once again evident. It was also possible to revert the *ras*-transformed mouse cells by introduction and expression of sense

constructs of a full length cDNA to this gene (S. Contente, unpublished data)..

This phenotypic suppressor of *ras* was subsequently identified as lysyl oxidase [15]. Lysyl oxidase is an extracellular, copper-dependent enzyme catalyzing the oxidative deamination of lysine residues in collagen and elastin to peptidyl α-aminoadipic-δ-semialdehyde. These aldehydes condense spontaneously with one another to form the covalent cross-links found in mature collagen and elastin [16]. Lysyl oxidase is known to be produced by aorta and dermal connective tissue as well as by fibroblasts, chondrocytes, and smooth muscle cells. Although lysyl oxidase mRNA was not detected in rat kidney, liver, brain, or heart tissue by RNA blot studies [17], immunohistochemical studies have localized lysyl oxidase to endothelial, basal, biliary epithelial and glomerular epithelial cells [18]. Lysyl oxidase might therefore have a function in cells that do not normally produce collagen or elastin. Lysyl oxidase activity was reduced in several human tumor cell lines of fibroblast origin, as well as in a choriocarcinoma and a melanoma [19].

Lysyl oxidase may possess other functions, as yet undefined, or may oxidize lysine in substrates other than collagen or elastin [20, 21]. Lysyl oxidase with enzymatic activity was recently localized to nuclei of smooth muscle cells [22]. It is possible that in the mouse system the oxidation of peptidyl lysine residues in precursors to fibrous collagen and/or elastin resulted in the decreased ability of cells to form tumors, despite the persistence of augmented expression of the *ras* oncogene. It is also possible that improperly cross-linked collagen and elastin molecules contribute to uncontrolled cell growth. There may also be a covalent interaction between p21 and lysyl oxidase in the cell (Philip Trackman, personal communication). This finding raises the possibility that a form of lysyl oxidase localized to the cytoplasm may play a direct role in the regulation of p21 function.

It therefore appeared that the overexpression of a non-mutated c-H-*ras* gene in mouse cells had a transforming effect that included the down regulation of the expression of lysyl oxidase. Furthermore, the restoration of lysyl oxidase message expression was not only associated with the reversal of the transformation, but it also was needed for the reversion to a non-tumorigenic cell line.

The purpose of the present work was to analyze the pattern and level of expression of the messenger RNA of lysyl oxidase in human breast tissue, both normal and neoplastic, and in established breast cell lines. Also, we wished to determine if an overexpression of normal *ras* in human breast cancer is accompanied by a decrease in the expression of lysyl oxidase, in a parallel with the situation in the mouse fibroblast model. The approach used for this portion of the work utilized the technique of reverse transcriptase-polymerase chain reaction (RT-PCR) for RNA extracted from cultured cell lines, and *in situ* RT-PCR on sections of fixed, paraffin-embedded breast tissue to determine in which cell types, if any, the messenger RNAs of lysyl oxidase and H-*ras* were expressed, and at what levels these genes were expressed. Finally, we

wished to determine if the transfection into human cancer cell lines of a lysyl oxidase expression plasmid could result in a restoration of normal cell growth and loss of tumorigenic potential.

BODY

Experimental Methods

Cell lines, cell culture and extraction of total cell RNA.

Cell lines were obtained from the American Type Culture Collection, Rockville, MD, and cultured in the supplemented medium and under the conditions specified by the supplier. Cell lines employed were: Hs578T and Hs578Bst, derived from a ductal carcinoma and normal marginal tissue, respectively [23]; BT-474 and BT-483, both ductal carcinomas [24]; T47-D, an infiltrating ductal carcinoma [25]; MCF-10a, a spontaneously immortalized epithelial line derived from mammary gland cells [26]; UACC-812, an infiltrating ductal carcinoma [27]; MCF-7, from a pleural effusion of an adenocarcinoma [28] that expresses fairly high levels of p21 derived from the N-*ras* gene [29]. MCF-7 does not express the endogenous H- or K-*ras* genes. Total cellular RNA was extracted from cultured cells using Trizol reagent (Life Technologies, Gaithersburg, MD), according to the manufacturer's instructions.

Reverse transcription and Polymerase Chain Reaction (RT-PCR)

First strand synthesis was performed using 2 μg of total cellular RNA, which had been heated at 75°C for 3 min, spun briefly and chilled on ice, in a total reaction volume of 30 μl containing 0.5 mM each of dATP, dCTP, dGTP, and dTTP, 0.01 M dithiothreitol, 20 units of RNasin (Promega, Madison, WI), 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 400 units of Superscript II RNase H$^-$ reverse transcriptase (Life Technologies), and 250-500 ng of gene-specific minus strand oligonucleotide primer. The mixture was incubated at 42°C, 1 hr; 75°C, 10 min, and stored on ice. For PCR, 3 μl of first strand reaction was mixed in a final volume of 100 μl containing 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 100 pmole each of plus and minus strand oligonucleotide primer, $MgCl_2$, and 2.5 units of Taq polymerase (Life Technologies). For hot start reaction, a mixture of all components except Taq polymerase were overlaid with mineral oil, and heated for 5 min at 94°C. Taq polymerase was then added, and the reaction cycled in a DNA Thermal Cycler (Perkin-Elmer, Foster City, CA) as follows: 24 cycles of 1 min, 94°C; 2 min, 58 or 60°C; 45 sec, 72°C, followed by 1 cycle of 1 min, 94°C; 2 min, 58 or 60°C; 10 min, 72°C. 10 μl of each reaction was analyzed by electrophoresis on a 2.5% agarose gel, blotted to a charged nylon membrane, and hybridized with a gene-specific oligonucleotide that had been labeled with γ-^{32}P ATP (DuPont NEN, Boston, MA) by polynucleotide kinase (Boeringher-Mannheim, Indianapolis, IN). Blots were hybridized for 18 hr at 42°C in 5 × SSPE (1 × SSPE is 0.15 M NaCl, 0.01 M NaH_2PO_4, 0.001 M EDTA, pH 7.4), 5 × Denhardt reagent (1 × Denhardt is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.1% $NaDodSO_4$, and

washed at room temperature in 5 × SSPE, 0.1% $NaDodSO_4$. Blots were imaged on XAR-5 film (Eastman Kodak, New Haven, CT), or on a Betascope 603 Blot Analyzer (Betagen, Waltham, MA). Oligonucleotides used for lysyl oxidase: HLOXPLUS, caccgcccgtcactggttccaag; HLOXMINUS, ccgccgcgcatctcaggttgtac; HLOXPROBE, cttccagtacggtctccc. For ras: HRASPLUS, gcctgttggacatcctggataccg; HRASMINUS, acttgcagctcatgcagccgg; HRASPROBE, ccagtacagggagcagatc. For G3PDH: GAPSENSE, ccatggagaaggctgggg; GAPANTISENSE, caaagttgtcatggatgacc; GAPPROBE, ctaagcatgtggtggtgca. For detection of adrenomedullin message, AMPLUS, aagaagtggaataagtgggct; AMMINUS, tggcttagaagacaccagagt; AMPROBE, ctggaagttgttcatgctctg. G3PDH was used as a positive control for RT-PCR on extracted RNA, and adrenomedullin was tested as a positive control for *in situ* RT-PCR on human breast cancer tissue sections.

Northern blot analysis.

10 μg of total cellular RNA was electrophoresed on a 1.2% agarose gel in 10 mM sodium phosphate, pH 6.8, containing 0.1 μg/ml of ethidium bromide [30], and transferred by capillary blotting overnight to a charged nylon membrane (Qiagen, Chatsworth, CA) using 20 × SSPE. A35, which is a 1.9 kb human lysyl oxidase cDNA, was used as a probe. 100 ng of gel-isolated cDNA was labeled with 50 μCi of ^{32}P-dCTP (DuPont NEN) using a random primer kit (Stratagene, La Jolla, CA). Blots were hybridized at 42°C in 50% formamide, 5 × SSPE, 5 × Denhardt reagent, 0.1% $NaDodSO_4$, 100 μg/ml sheared, denatured herring sperm DNA, for 18 hours. Blots were washed twice with 2 × SSPE, 0.1% $NaDodSO_4$ at room temperature, and twice with 0.1 × SSPE, 0.1% $NaDodSO_4$ at 50°C, and imaged as described above.

Preparation of tissues and sections.

All specimen handling and subsequent procedures were carried out under conditions that minimized the possibility of contamination by RNases. Deionized, distilled water that had been treated with 0.1% diethylpyrocarbonate for 18 hrs and then autoclaved (DEPC-water) was used. All solutions, equipment, and glassware used during all phases of the protocol were appropriately treated to remove nucleases. Normal and abnormal human breast tissues, skin, and aorta that were obtained by surgical removal or at autopsy were received as formalin-fixed, paraffin-embedded blocks. Series of sections of 4-5 μm were cut and mounted on silanated slides. Silanated slides were purchased (DIGENE Diagnostic Laboratories, Silver Spring, MD) or prepared by treating plain clean slides with a solution of 2% 3-aminopropyltriethoxysilane (AEC) (Sigma, St. Louis, MO) in acetone, followed by washing with 1 l of DEPC-water.

Preparation of paraffin embedded, cultured cell sections.

Cultured cells were used for positive and negative controls. Cell line FS4 (human foreskin fibroblast) was used for lysyl oxidase message, RS485 (c-H-*ras*-transformed mouse fibroblast) was used for c-H-*ras* message, and NIH 3T3 (mouse fibroblast) was used for a c-H-*ras* negative control. A total of 6-8 × 10^6 cells at 60-80% confluence were collected after trypsinization, and washed thrice with DEPC-treated 1 × phosphate buffered saline (PBS). The supernatant was completely removed by aspiration, and the cells were fixed in 10% buffered formalin for 24-48 hrs. The fixed cells were then embedded in paraffin (American Histopathology Laboratory, Rockville, MD), and 4-5 μm sections were cut and mounted on silanated slides.

Fixation of sectioned tissue samples for *in situ* RT-PCR.

Slides containing sectioned tissue samples were placed at 80°C under vacuum for 1 hr to remove remaining paraffin wax, and then treated for 4 hr at room temperature in a freshly prepared solution of 4% paraformaldehyde in DEPC-treated 1 × PBS. Slides were then washed for 10 min once with 3 × PBS and twice with 1 × PBS. Slides not to be used immediately were dehydrated in 100% ethanol and stored at -80°C for future use.

Proteinase K treatment.

The optimal concentration of proteinase K and treatment time was standardized for each type of tissue. Paraformaldehyde treated tissue sections, if previously dehydrated and stored at -80°C, were first rehydrated for 2 min in 350 ml of each of a series of graded ethanols (absolute, 95%, 70% and 50%). The slides were then placed in a humidity chamber (Snap Box, Andes Scientific, Franklin Square, NY), a solution of proteinase K (Fluka Chemical Corp., Ronkonkoma, NY) was applied in a volume sufficient to cover the entire tissue section, usually 250 μl, and the chamber was placed at 37°C. The concentration of proteinase K was first titrated for different tissues. For sections of skin and aorta, 20 μg/ml of proteinase K for 15 min at 37°C did not damage the morphology of the tissue. For normal breast tissues, cell morphology was preserved after treatment for 15 min at 37°C using a 10-15 μg/ml concentration of proteinase K. For different types of abnormal breast tissue sections, there was no generalized concentration of proteinase K or incubation time that was universally applicable. Optimal treatment for these sections was achieved at between 5-10 μg/ml of proteinase K for between 7-10 min. Following the proteinase treatment, enzyme was inactivated by immersion of the slides in 0.1 M glycine in 1 × PBS for 5 min. Slides were then washed thrice in 1 × PBS and dehydrated for 2 min in each of 50%, 70%, 95% and absolute ethanol.

Digestion of cellular DNA

Depending upon the size of the tissue section, between 50-100 μl of RQ1 RNAse-free DNAse

(Promega, Madison, WI), diluted with DEPC-water to 600-750 U/ml, was placed onto the sections. These were immediately covered by a rectangular piece of parafilm that was slightly larger than a size of the tissue, using the side of the film that had been protected by paper. The slides were then incubated for 12-16 hr at room temperature (25-28°C) in the humidity chamber. The parafilm coverslip was then carefully removed from the top of the each tissue section with a nuclease free, sterile forceps. The slides were then washed twice with DEPC-treated 1 × PBS and dehydrated in graded ethanols as described above.

Reverse transcription in tissue sections

The cellular messages for lysyl oxidase, H-*ras*, or adrenomedullin in the treated tissue sections were reversed transcribed *in situ*. A 60 µl reaction mixture containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM each of dATP, dGTP, dCTP, and dTTP, 20 units RNasin (Promega), 0.01 M dithiothreitol, 400 units Superscript II RNase H^- reverse transcriptase (Life Technologies) and 500 ng of antisense (minus) primer was placed onto the tissue section, covered with a fresh piece of parafilm as described above, and sealed to the slide using rubber cement. The slides were then incubated at 42°C for 1 hr, 70°C for 10 min in a PTC-100-16MS Slide Cycler (M-J Research, Watertown, MA), quickly removed and placed on ice for 1 min. The parafilm was then removed, and the slides washed and dehydrated as described above for DNase treatment. Negative control slides for the RT reaction were processed using 60 µl of reaction mixture without RT enzyme. All water used in RT and PCR reactions was first sterilized by filtration through a 0.22 µm filter, followed by UV irradiation for 15 min. Aerosol-barrier pipet tips were used for all manipulations.

Polymerase Chain Reaction (PCR) in tissue sections

The cDNAs synthesized *in situ* were amplified *in situ* using either unlabeled deoxynucleotide triphosphates (indirect detection method) or digoxigenin-11-dUTP (Boehringer-Mannheim, Indianapolis, IN) (direct detection method). For the indirect detection method, 30-40 µl of a PCR reaction mixture containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 3 µg/ml bovine serum albumin, 100 pmole each of plus and minus oligonucleotide primers, 0.5 units/ml Taq DNA polymerase (Life Technologies), and 0.2 mM each of dATP, dCTP, dGTP and dTTP was placed onto the tissue section, covered with a 20 × 30 mm glass coverslip (Bellco Glass, Vineland, NJ), and carefully sealed with rubber cement to prevent evaporation during cycling. A hot-start reaction was achieved by pre-complexing 5 units of Taq polymerase with 1.1 µg of Taqstart™ antibody (Clontech, Palo Alto, CA) in a final volume of 20 µl containing 50 mM KCl, 10 mM Tris-HCl, pH 7.0, for 5 min at 22°C. For the direct detection method, a 1:19 digoxigenin-11-dUTP:dTTP solution was substituted for dTTP in the PCR reaction mixture. Slides amplified for the direct detection method were not pretreated with DNase, because such treatment creates small DNA fragments that could be amplified, resulting in artifactual signals.

Slides were cycled as follows. For lysyl oxidase, 2 min, 96°C, followed by 30 cycles of 1 min, 94°C; 2 min, 60°C; 45 sec, 72°C, followed by 1 cycle of 1 min, 94°C; 2 min, 60°C; 10 min, 72°C. For *ras*, 2 min, 45 sec, 96°C, followed by 30 cycles of 1 min, 94°C; 1 min, 57°C; 50 sec, 72°C; followed by 1 cycle of 1 min, 94°C; 1 min, 57°C; 10 min, 72°C. For adrenomedullin, 2 min, 45 sec, 96°C, followed by 30 cycles of 15 sec, 94°C; 15 sec, 56°C; 1 min, 72°C; followed by 7 min, 72°C. After cycling, slides were washed twice for 5 min in 1 × PBS and dehydrated in graded ethanols as described above.

Detection of *in situ* RT-PCR products

For indirect detection of unlabeled RT-PCR products, slides were hybridized with a digoxigenin-labeled oligonucleotide probe. For lysyl oxidase and *ras*, this probe was chosen so that it spanned an exon-intron junction, ensuring that hybridization could only be with message-derived products, and not with fragments of genomic DNA. The oligonucleotides to be used as probes were first purified by HPLC and then labeled at the 3' terminus with digoxigenin-11-ddUTP, using terminal transferase (Genius™ 5, Boehringer-Mannheim) according to the manufacturer's instructions. After labeling, the probe was purified by precipitation with 2.5-3.0 volumes of chilled ethanol, in the presence of 0.4 M LiCl and 20 μg glycogen (Boehringer-Mannheim), for 30 min at -80°C. The probe was then thawed briefly at room temperature, centrifuged at 13,000 × g for 15 min, and the pellet was washed twice with 100 μl of 70% ethanol. The pellet was then centrifuged at 13,000 × g for 5 min, and remaining ethanol was completely removed. The pellet was dried and resuspended in water, and stored at -20°C. The yield of the probe was estimated by comparison with the standard template provided, and the concentration was measured by UV absorption at 260 nm. 30 μl of hybridization solution (5 × SSPE, 5 × Denhardt reagent, 0.1% $NaDodSO_4$) containing 30-60 ng of probe was placed on to the each slide, covered with a 20 × 30 mm glass coverslip and sealed with rubber cement. The slides were heated at 98°C in the slide cycler for 15 min, and hybridization was continued for 12-16 hr at 37°C in an incubator. The coverslip was then removed and slides were washed in 0.2 × SSC at 48°C for 15 min, followed by two washes in 1 × PBS for 5 min each.

Detection of *in situ* PCR products.

Following either the direct incorporation of digoxigenin by Taq polymerase, or hybridization of a digoxigenin-labeled oligonucleotide probe with the unlabeled PCR product, slides were placed in a blocking solution of 50 mg/ml bovine serum albumin in 100 mM Tris-HCl, pH 7.8, 150 mM NaCl, 150 mM $MgCl_2$ for 10 min. Anti-digoxigenin-alkaline phosphatase antibody (Boehringer Mannheim, Indianapolis, IN) was diluted 1:250 in 1 × PBS, and 0.1-0.2 ml was added to the slide and incubated for 2 hr at room temperature in the humidity chamber. The slides were then washed twice with 1 × PBS containing 0.2% Tween 20 to remove excess antibody, and treated for 10-15 min with 100 mM Tris-HCl pH 9.5, 150 mM NaCl, 50 mM $MgCl_2$ (alkaline buffer

solution). Subsequently, the slide was carefully wiped with a lint-free towel, without touching the tissue section, and placed in a humidity chamber. A freshly prepared substrate solution containing 275 mg/ml nitro-blue-tetrazolium, 50 mg/ml 4-bromo-5-chloro-3-indolylphosphate, 1 µl/ml Levamisol (Vector Laboratories, Burlingame, CA) in alkaline buffer solution was placed onto the tissue, and the chamber was placed in the dark for the development of the purple-blue color signal. The reaction was stopped by transferring the slides into a solution containing 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, after observing under the microscope for the proper intensity of the color signal. This development time was standardized to 16 hr for indirect detection and 2-3 hr for direct detection. Excess background was removed by dipping the slides for 5-7 min in 95% methanol. Finally, the slides were mounted using either Aquamount or Crystal mount (Biomeda-Fisher, Pittsburgh, PA), examined using an Olympus Model BX40 light microscope and photographed using an Olympus PM20 automatic photomicrographic system with 35 mm Gold 200 color film (Eastman Kodak).

RESULTS AND DISCUSSION

Task 1. Analyze lysyl oxidase message and *ras* message expression in human breast cell lines.

Preliminary RT-PCR studies on RNA extracted from normal cell line Hs578Bst indicated that lysyl oxidase mRNA was expressed abundantly in that line, indicating that this gene is expressed in at least one normal breast cell type. Hs578Bst was derived from normal tissue peripheral to a ductal carcinoma, may be myoepithelial in origin, and has a fibroblast-like morphology. The expression of lysyl oxidase message in RNA extracted from Hs578T, the tumor-derived line from the same patient, was about 4% of that observed for Hs578Bst. RNA blot analysis is frequently used to determine if a specific message is expressed in a cell line or tissue type, however, the limit of detection for this method is about 5 pg, and rare messages may not be detected. The method of reverse transcriptase-polymerase chain reaction (RT-PCR), which is more sensitive, is useful to detect such rare messages or confirm a negative result from an RNA blot analysis. RNA blots were used first to both examine the quality of the RNA prepared and to see if lysyl oxidase message could be detected in the five RNA preparations then available. No lysyl oxidase signal was detected in lines T47-D, MCF-7 or BT-474; message could be detected in Hs578T, and at low levels in MCF-10a. In order to confirm this lack of expression of lysyl oxidase message, RT-PCR was performed (Appendix 1, Fig. 24) . The expected PCR product of 365 bp, derived from the lysyl oxidase reverse-transcribed message, was detected in only two transformed lines. As expected, message was highly expressed in the normal line Hs578Bst, and was also detected, in much lower amounts, in Hs578T. There was also a PCR product just visible for MCF-10a. No message could be detected in T47-D, MCF-7, BT-474, UACC 812, or BT-483. The expected RT-PCR product of 405 bp for the H-*ras* message was just detectable in Hs578Bst, MCF-10a, and BT-483, present in small amounts in T47-D, MCF-7, BT-474, and

UACC 812, and present in large amount in Hs578T, which is known to have a codon 12 mutation in the H-*ras* gene [2]. As a positive control, RT-PCR for glyceraldehyde-3-phosphate hydrogenase (G3PDH) message was also performed. The expected 195 bp PCR product was detected in large amount in each of the RNA preparations. With the exception of Hs578T, there did not appear to be an elevated level of expression of H-*ras* message in the seven established tumor cell lines examined, although compared with the normal Hs578Bst, more *ras* mRNA was present in T47-D, MCF-7, BT-474, and UACC 812. Therefore, at least in the group of cell lines examined here, the lack of expression of lysyl oxidase mRNA did not generally correspond with an elevated expression of *ras* mRNA.

RNA from cell lines MCF10A ("normal") and MCF10AneoT (MCF 10A transformed by mutated *ras* oncogene. Cell line is tumorigenic in mice) was kindly provided by Dr. S. Wolman. RT-PCR was performed using lysyl oxidase and ras primers. Results for this MCF10A RNA were the same as observed previously with this cell line: PCR products of the expected sizes for lysyl oxidase and for *ras* were visible but not abundant when analyzed on agarose gels, and hybridized specifically with the appropriate radiolabeled probes. In the *ras*-transformed MCF10AneoT RNA, PCR product for *ras* was detected, but lysyl oxidase product was undetectable. This result conforms with the prediction that cellular transformation by *ras* oncogene leads to loss of lysyl oxidase gene expression.

Task 2. Analyze pattern of lysyl oxidase and *ras* message expression in tissue sections of normal and cancerous human breast.

Expression of lysyl oxidase message and *ras* message in embedded cells and human skin and aorta sections.

Preliminary studies were performed on sections of human and mouse cells grown in culture and embedded in paraffin, and on sections of paraffin-embedded human skin and aorta. The purposes of these studies were (1) to develop and perfect all facets of the technique of *in situ* RT-PCR, (2) to determine the optimal conditions for reverse transcription, for PCR cycling, and for *in situ* hybridization of oligonucleotide probes, and (3) to verify that the oligonucleotides selected would amplify messages in cells or tissues known to express the genes.

Signal was detected for human lysyl oxidase in fixed, embedded FS4 cells, after *in situ* RT-PCR, using a digoxigenin-labeled oligonucleotide probe (indirect detection). The signal was distributed throughout the cytoplasm of the cells. Similarly, RT-PCR products from *ras* mRNA could be amplified in fixed, embedded RS 485 cells, and were visualized as cytoplasmic signal. In normal human skin, lysyl oxidase was found to be expressed, as expected, in epidermal and basal cells. Signal could also be detected in sections of aorta, in which lysyl oxidase is known to be abundantly expressed.

Expression of lysyl oxidase message and *ras* message in human normal breast tissue sections.

Sections of normal breast tissue were subjected to RT-PCR *in situ* using lysyl oxidase or *ras* primers. Both the direct and indirect detection methods were employed. Signal for lysyl oxidase was found localized to the epithelial cells lining ducts and acini, and was also detectable in stromal fibroblasts (Appendix 1, Figs.1-4). Comparable results were obtained with both detection methods although the signal was more intense for the direct method. The signal obtained after amplification with *ras* primers was also localized to epithelial cells lining ducts and acini (Appendix 1, Figs. 5-8) , and was also more intense by the direct method. Therefore, in normal breast, both lysyl oxidase and *ras* messenger RNA are expressed, and each can be found in epithelial cells lining duct structures. As a negative control for lysyl oxidase in normal tissue, tissue sections were subjected to RT-PCR using reaction mixes lacking reverse transcriptase and Taq polymerase. No signals were detected in the negative control (Appendix 1, Fig. 9). A hematoxylin-eosin (H&E) stained section of normal breast (Appendix 1, Fig.10) is provided for reference. Adrenomedullin is a hypotensive peptide [31], believed to be a new circulating hormone, which has been found in a number of tissues, including breast. Adrenomedullin was investigated as a possible positive control for *in situ* RT-PCR on breast tissue sections. Adrenomedullin primers were used to amplify message in normal breast tissue sections, and signal for adrenomedullin was found localized to ductal and acinar epithelial cells. However, adrenomedullin message expression was found to be reduced in ductal carcinoma *in situ*, and signal was observed only in patchy areas of neoplastic cells close to the edges of ducts. Due to the inconsistent expression of this marker in the tumor tissue, its use as a positive control was discontinued.

Expression of lysyl oxidase message and *ras* message in tumor tissue sections.

Ductal carcinoma *in situ* (DCIS) and infiltrating ductal carcinoma (IDC).
Several cases of DCIS, IDC and mixed DCIS-IDC were examined by *in situ* RT-PCR. The expression of lysyl oxidase in cases of ductal carcinoma was variable. In some patients, lysyl oxidase message was either not expressed, or expressed at very low levels, in the cancerous ducts and in infiltrating nests of cells (Appendix 1, Fig. 11,13,14). In normal-appearing ducts located in other areas of the same tissue, lysyl oxidase expression was retained (Appendix 1, Fig. 12). Additionally, staining for lysyl oxidase message in a number of stromal fibroblasts was detected in cases where lysyl oxidase expression was undetectable in the neoplastic cells, indicating that the lack of expression detection was not artefactual. In other cases of ductal carcinoma, the lysyl oxidase message continued to be expressed in the cancerous ducts and infiltrating cells. This expression was sometimes patchy in nature throughout the sample. The expression of *ras* message was consistently abundant in the neoplastic cells of all cases of DCIS/IDC that were examined (Appendix 1, Figs.15-16). Negative controls (Appendix 1, Fig.17) were always

performed, and no staining for lysyl oxidase was observed. An example of DCIS (H&E staining) is provided for reference (Appendix 1, Fig. 18).

Lobular carcinoma *in situ* (LCIS) and infiltrating lobular carcinoma (ILC).

Cases of LCIS and ILC were less available for study; a case provided with a diagnosis of LCIS and ILC was judged by another pathologist to be a mixture of ductal and lobular carcinomas. The signal detected for both lysyl oxidase (Appendix 1, Fig. 20) and *ras* (Appendix 1, Fig. 22) messages in areas of LCIS was comparable to that found in normal tissue. Signal was also detected in invasive areas for both lysyl oxidase (Appendix 1, Fig. 19) and *ras* (Appendix 1, Fig. 21). Negattive controls exhibited no staining for lysyl oxidase (Appendix 1, Fig. 23) There did not appear to be any decreases in lysyl oxidase expression in these cases of LCIS/ILC, as was observed in some cases of DCIS. However, not enough cases could be examined for valid conclusions to be drawn.

Expression of lysyl oxidase in other breast conditions.

Many of the tissue samples contained areas diagnosed as ductal hyperplasia or fibrocystic regions. In cases exhibiting ductal hyperplasia, the expression of lysyl oxidase appeared reduced from that observed in normal ducts, while the expression of *ras* was as abundant as in normal tissue. Fibrocystic lesions were present in at least two cases analyzed. The fibrocystic ducts in the abnormal areas of the tissue showed an intense expression of lysyl oxidase. The expression level for c-H-*ras* in the fibrocystic areas was unchanged from that observed in areas of normal breast tissue in the same sample. Fibrocystic areas might be expected to exhibit an increased expression of lysyl oxidase, as the function of the enzyme is catalysis of collagen cross linking.

Improving and refining the *in situ* RT-PCR (ISRTPCR) technique.

1. Use of Fast Red (Boeringher Mannheim) in place of NBT-BCIP as a substrate for detection of lysyl oxidase and *ras* RT-PCR products.

Rationale: Fast Red gives a brilliant red color signal, and is a substrate for alkaline phosphatase. Alkaline phosphatase is the enzyme conjugated to the anti-digoxigenin antibody used to detect PCR products in this protocol. The cell cytoplasm would be stained red for positive signals, as opposed to blue with NBT-BCIP. An excellent contrast to Fast Red would then be obtained using a Hematoxylin counterstain, which imparts a blue color to cell nuclei. This counterstain is important in the ability to distinguish tissue architecture under the microscope.

Results: When direct ISRTPCR was employed (in which digoxigenin-labeled nucleotides are incorporated into the PCR product), results were excellent in terms of the color intensity of the stained regions. When indirect ISRTPCR was employed (in which the PCR products are unlabeled and then subsequently hybridized with a digoxigenin-labeled probe specific for the PCR product), the staining intensity of the Fast Red signal was much lower. Even an overnight incubation with the Fast Red substrate did not increase the intensity of the signal.

Conclusions: Because direct ISRTPCR was found to be less reliable in terms of false positive results, we decided to use only the indirect method. Therefore, the use of Fast Red substrate for future indirect ISRTPCR studies was discontinued.

2. Use of Nuclear Fast Red (Vector Laboratories) as a nuclear counterstain.

Rationale: This would produce an excellent contrast with the blue color obtained in the cytoplasm using NBT-BCIP substrate to detect RT-PCR products.

Results: Initial experiments showed a non-specific stippled staining pattern. The difficulty was with the staining solution, which needs to be filtered before use. After filtration, specific red staining of nuclei with good contrast to the blue cytoplasm was obtained.

Conclusions: Nuclear Fast Red is a good counterstain and will be useful in future experiments. It should be filtered before each use.

3. Generation of longer digoxigenin-labeled probe using PCR

Rationale: The signal strength achieved with an oligonucleotide probe end-labelled with digoxigenin is limited. To increase the signal strength, a longer probe will be created from cDNA templates using PCR that incorporates digoxigenin-11-dUTP. This will create a probe containing more digoxigenin molecules.

Results: A 238 base pair probe for lysyl oxidase was generated using PCR including a digoxigenin-11-dUTP nucleotide. This probe hybridized specifically with the 365 base pair lysyl oxidase PCR product produced by the primers used in RT-PCR reactions. When compared with a standardized digoxigenin probe, this probe was shown to contain 50 pMoles/µl of digoxigenin, which is a high yield. After hybridization using the PCR-generated probe, the time needed for signal detection was shortened from 24-48 hours to 2-3 hours. This type of probe was also cost effective, as the cost of the PCR reaction reagents was less than those needed for 3' end labeling.

Conclusions: Longer, PCR-generated digoxigenin-labeled probes could be used in place of shorter, 3'-labeled oligonucleotide probes in future experiments.

4. Evaluation of "EZ" protocol, one-step RT-PCR employing rTth DNA polymerase (Perkin-Elmer).

Rationale: Use of one enzyme for both RT and PCR would reduce costs and save time and effort in each experiment.

Results: This protocol worked well with lysyl oxidase primers with an optimal annealing temperature of 60°C. For *ras* primers, the optimal annealing temperature is 57°C, which is three degrees below the optimum temperature for the rTth enzyme. Under these sub-optimal conditions for RT-PCR of *ras*, the results obtained were not comparable to those obtained for lysyl oxidase.

Conclusions: The results obtained with the lysyl oxidase primers were consistent over four experiments. This appears to be an excellent protocol. To employ this protocol, different primers for the *ras* message, having an optimal annealing temperature of 60°C or above, would need to be designed.

5. Evaluation of plastic "hybriwell" cover slips (PGC Scientifics) in place of glass coverslips for RT and PCR reactions.

Rationale: These plastic coverslips self-seal to the glass slide, and have a port for adding and removing reagents. They were developed for *in situ* hybridization protocols. Use of this type of coverslip avoids the sealing on of a glass coverslip, using rubber cement, twice during the protocol. This saves time and eliminates potential handling errors.

Results: The cover slips attached and remained well on the slide, however, due to the small volumes used in the RT and PCR steps, the distribution of reagent liquid over the slide was not uniform under the flexible plastic cover. There was uneven signal detected in control slides after the hybridization step, leading to the conclusion that the RT and/or PCR reaction was not uniform over the surface of the tissue.

Conclusion: Glass cover slips, although more difficult to handle, are superior in ensuring even distribution of RT-PCR reagents over the surface of the tissue, and will be employed in future experiments.

6. Comparison of reagents/kits for RT and PCR manufactured by Life Technologies, Perkin Elmer, and Clontech.

Rationale: Use of the best and most reliable reagents will ensure consistent results and conserve manpower.

Results: Reverse transcriptases and Taq polymerases from Life Technologies, Clontech, the Perkin Elmer RNA PCR Kit, and Perkin Elmer AmpliTaq Gold were all equally effective in ISRTPCR with either lysyl oxidase or *ras* primers. Hot starts using Taq-start antibody complex (Clontech) were comparable to those using AmpliTaq Gold Perkin-Elmer). Retesting of the EZ RTPCR Kit (Perkin Elmer), which employs rTth DNA polymerase, confirmed that this method worked well only with lysyl oxidase primers.

Conclusions: Use of the original method, with separate steps for RT at 42°C, and PCR with either hot start method gave consistent and reproducible results. Other investigators reported that the quantity of RT-PCR product was consistently reduced by increasing the RT reaction temperature above 50°C, as is required in the EZ protocol. In light of this, and the need to design new primers in order to use this method, this protocol was not adopted.

Task 3. Expression of human lysyl oxidase cDNA in human breast cancer cell lines

A mammalian expression plasmid construct, containing a full length human lysyl oxidase cDNA in the sense orientation, was used to transfect cell line BT474, using a liposome mediated method (Lipofectamine, Life Technologies). As a control, the vector without cDNA insert was transfected separately. The vector (pβApr-1-neo) is a 10 kilobase plasmid that carries a gene for neomycin resistance, selectable in mammalian cells using G418 antibiotic. The results of this transfection were poor. No $G418^r$ colonies could be isolated after transfection with vector alone or vector containing cDNA. The large size of the vector may have been a factor, also, the BT474 cell line may be difficult to transfect. In addition, this vector was not amenable to easy cloning of an antisense orientation lysyl oxidase cDNA for use as a control. Therefore, new constructs were created using the 3.5 kilobase mammalian expression vector pZeoSV2 (Invitrogen). Even with a fairly large insert, the construct will be small enough for efficient transfection. This vector contains the SV40 promoter to drive expression, carries a gene for Zeocin resistance, which is selectable in both bacteria and mammalian cells, and has many restriction enzyme sites available for subcloning. Human lysyl oxidase cDNA was cloned into this vector in both the sense and antisense orientation, and each construct was used to transfect cell line Hs578T. After selection with Zeocin, about 50 each sense and antisense colonies developed. All of the sense colonies, and 21 of the antisense colonies were picked, using trypsin soaked filter paper, and subcultured in medium containing Zeocin. Only three sense and two antisense colonies were successfully subcultured. These cell lines were expanded. The cell lines containing the sense lysyl oxidase expression construct had a flatter appearance in culture, and grew much more slowly than the antisense containing cell lines. RNA was isolated from the cell lines and analyzed by northern blot. Message of the size expected for the human lysyl oxidase cDNA (2 kb) was not detected in the sense cell lines after hybridization with radiolabeled probe. The transfection was repeated to obtain more sense colonies for analysis. Cloning cylinders were used instead to trypsin-soaked filter papers to try to improve the success rate in isolating colonies. No Zeocin-resistant colonies were successfully subcultured with this method.

CONCLUSIONS

Lysyl oxidase gene expression is absent or reduced in breast cancer cell lines. Five cell lines derived from human breast cancer did not express lysyl oxidase mRNA; another cell line expressed lysyl oxidase mRNA at about 4% of the expression observed in a paired normal cell line. A spontaneously immortalized line derived from a mammary gland cell (MCF 10A) expressed lysyl oxidase at low levels. H-*ras* gene expression varied in breast cancer cell lines; its expression level did not correlate with lysyl oxidase gene expression. Therefore, while breast cancer cells appear to lose expression of lysyl oxidase, this is not neccessarily accompanied by an increase in the expression of *ras*.

Transformation of MCF10A, which expresses lysyl oxidase at low levels, by a mutated *ras* oncogene resulted in the loss of lysyl oxidase expression. This is consistent with previous data in mouse cells, which lost lysyl oxidase expresion after cell transformation by *ras*.

Using the technique of *in situ* RT-PCR, lysyl oxidase was shown to be expressed in normal breast tissues. Message was expressed in ductal and acinar epithelium, as well as in some stromal fibroblasts. Message was also observed in endothelial cells and some lymphocytes. The finding of lysyl oxidase mRNA expression in normal ductal and acinar epithelial cells was consistent within tissue samples and was repeatable in independent samples. This finding is completely opposed to that reported by Peyrol, *et al.* [32], who found no lysyl oxidase message expression in normal breast epithelium.

Lysyl oxidase expression was reduced in cases exhibiting ductal hyperplasia (DH). DH is often considered to be a precursor condition for breast carcinoma. However, the expression of lysyl oxidase in the neoplastic cells of DCIS and IDC was not always reduced. In some cases, there was no expression of lysyl oxidase in either in situ or infiltrating cells; normal ducts in the same tissue samples did show lysyl oxidase expression. In other cases, lysyl oxidase expression in neoplastic cells was reduced but not absent; in some, expression was not reduced and appeared comparable to that observed in normal controls. Therefore, loss of lysyl oxidase message expression is not simply correlated with ductal neoplasia, and may also depend on another variable, such as hormone receptor status.

No changes in lysyl oxidase message expression were observed in cases of LCIS/ILC, however, fewer cases of lobular carcinoma were available for study. The continuance of gene expression in this type of carcinoma might reflect differences in etiology.

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Localization of lysyl oxidase and *ras* message in normal and neoplastic breast tissues using *in situ* RT-PCR. "USUHS Research Day" 28-29 March 1996, Bethesda, MD

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The expression of lysyl oxidase, a phenotypic suppressor of *ras*, in breast cancer. AACR " Cancer Prevention and Therapy " 13-18 February 1995, Kaanapali, HI

Personnel Receiving Pay From This Effort

Thaker, Vishakha B.

Appendix 1

Indirect *in situ* RT-PCR for lysyl oxidase. Normal breast tissue.

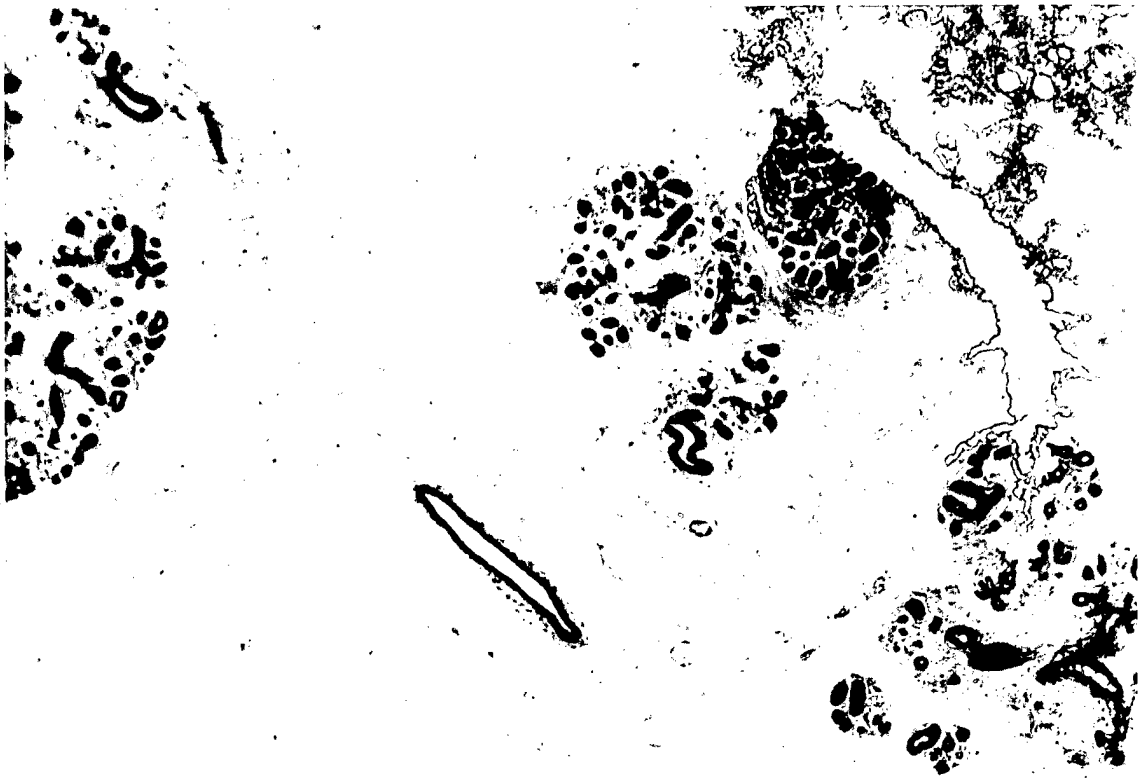


FIG. 1
10X Mag.

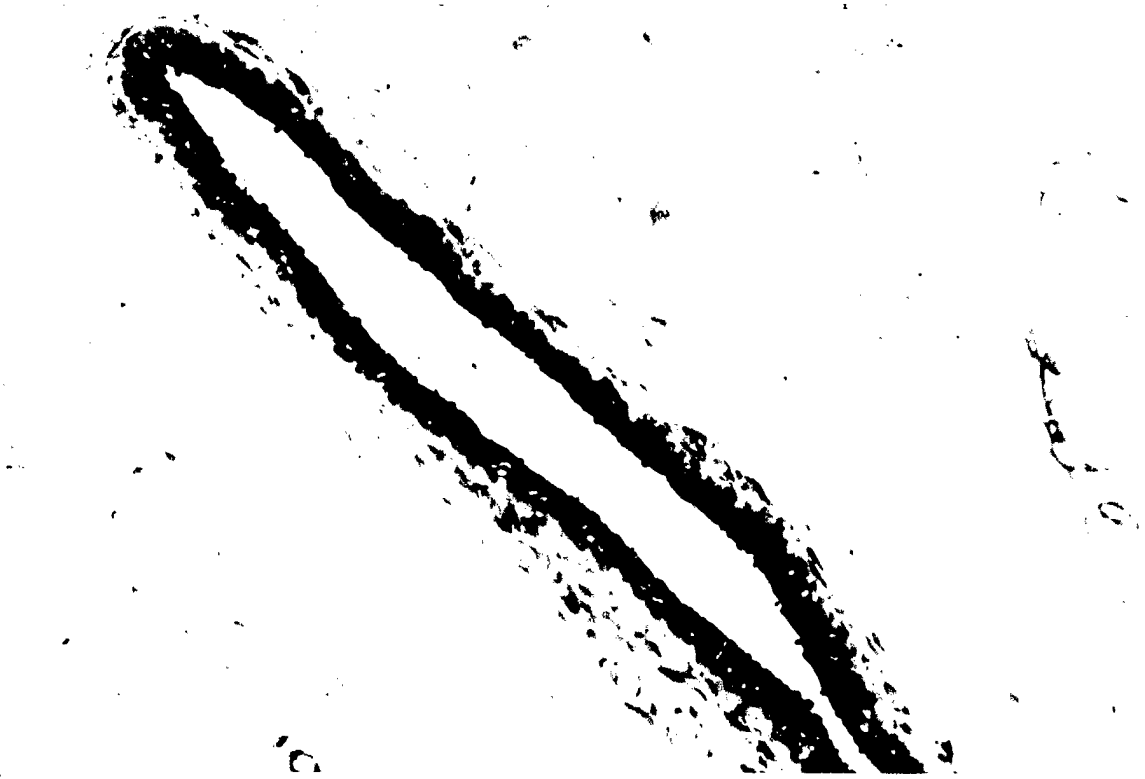


FIG. 2
50X Mag.

Appendix 1

Indirect *in situ* RT-PCR for lysyl oxidase. Normal breast tissue.

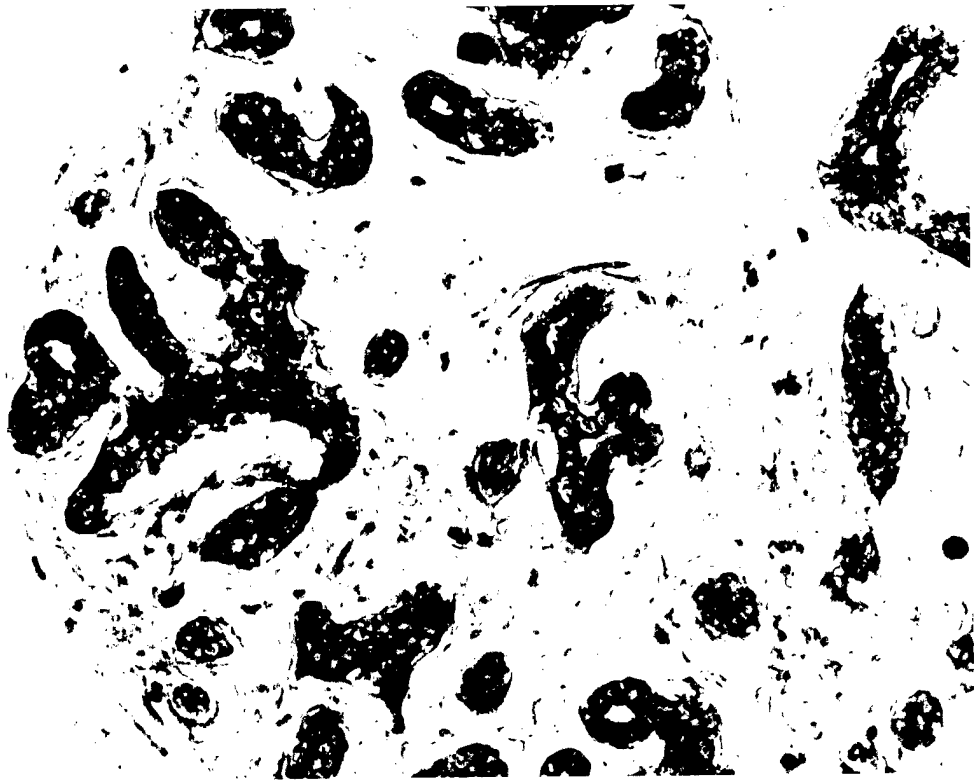


FIG. 3
50X Mag.

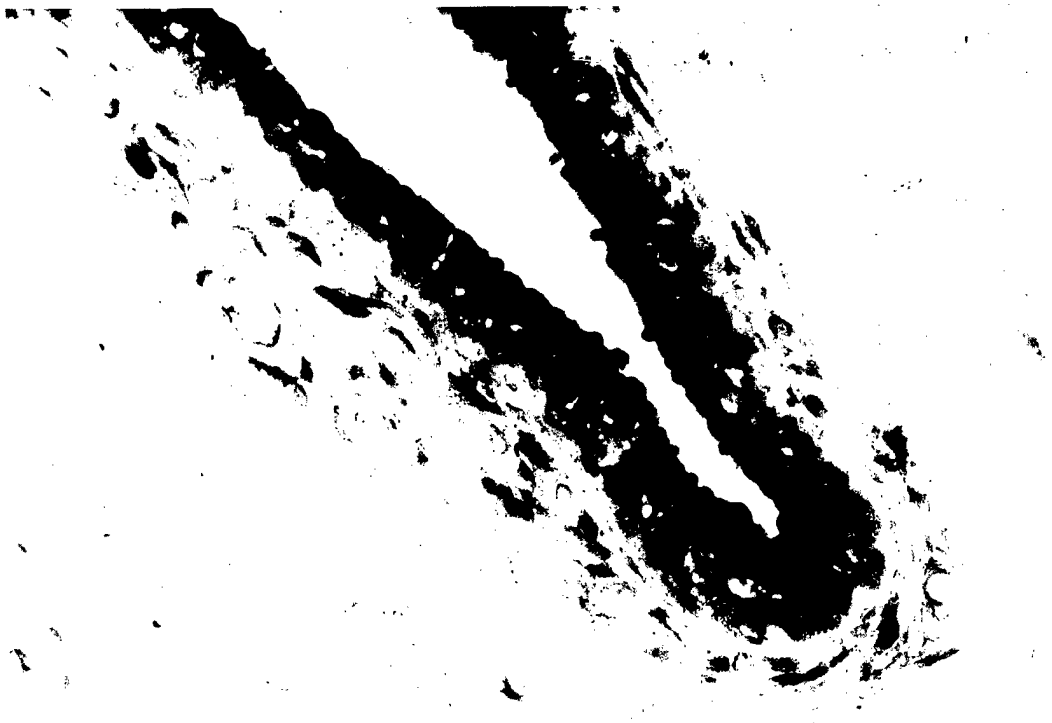


FIG. 4
100X Mag.

Appendix 1

Indirect *in situ* RT-PCR for *ras*. Normal breast tissue.

FIG. 5
25X Mag.

FIG. 6
25X Mag.

Appendix 1

Indirect *in situ* RT-PCR for *ras*. Normal breast tissue.

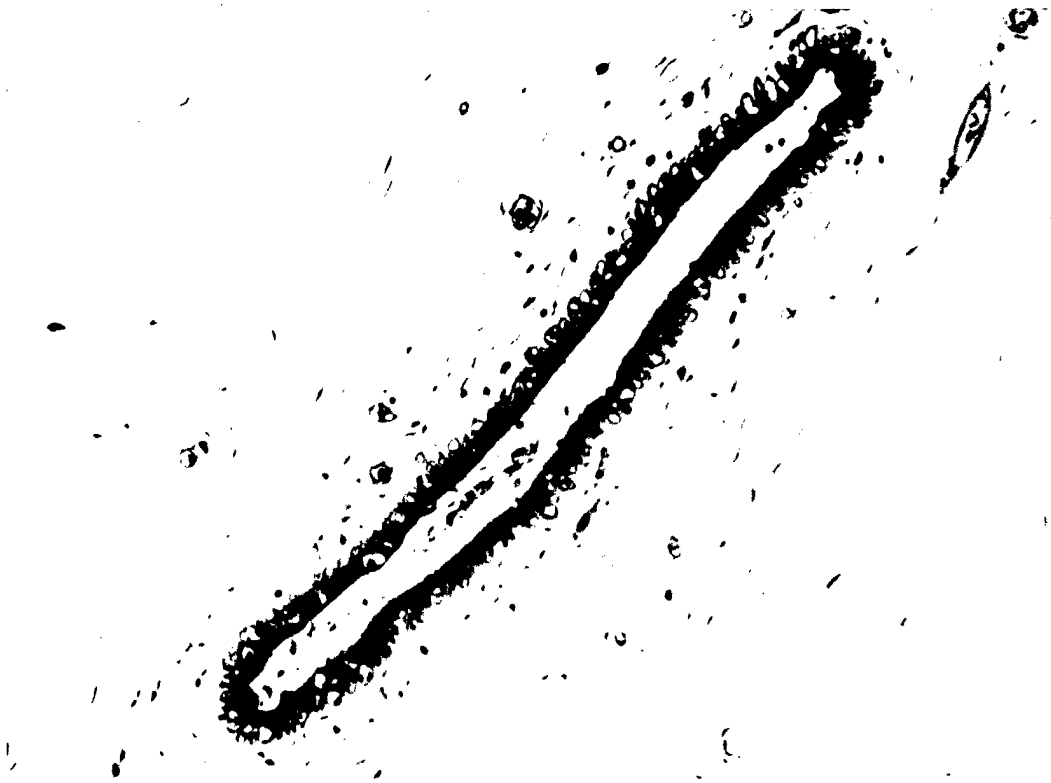


FIG. 7
50X Mag.

FIG. 8
100X Mag.

Appendix 1

Negative control (lacking RT and Taq): Indirect *in situ* RT-PCR for lysyl oxidase. Normal breast tissue.

FIG. 9
25X Mag.

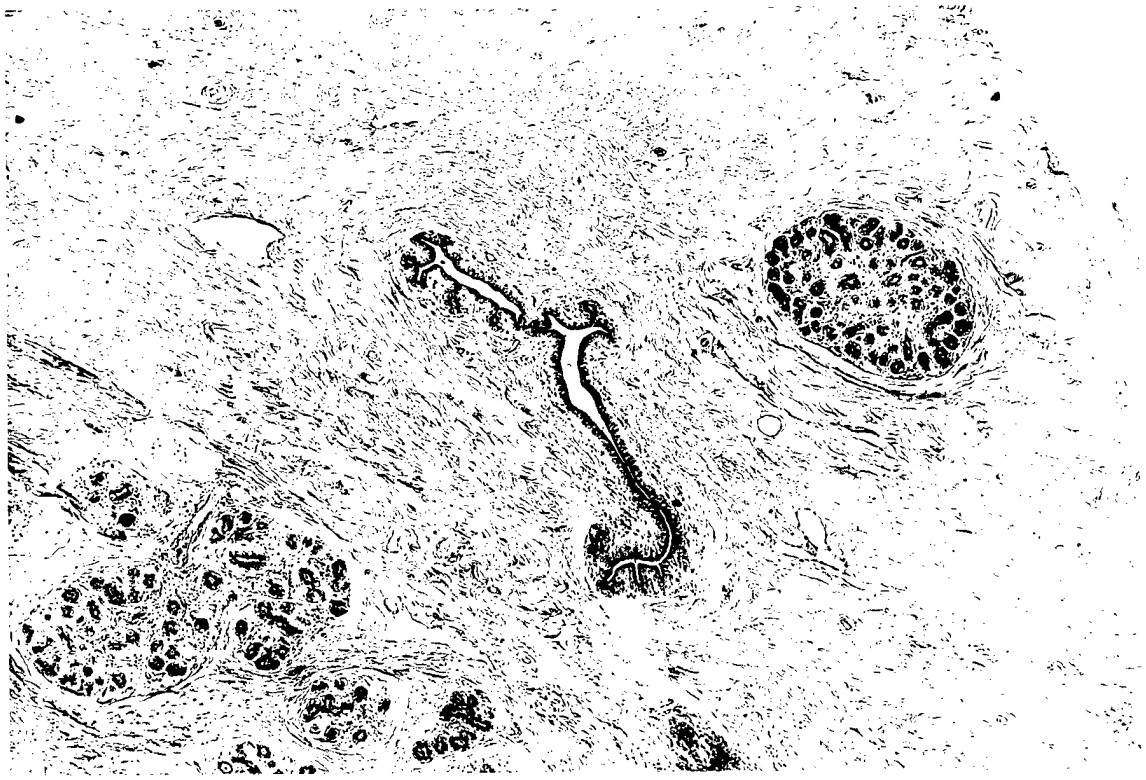


FIG. 10
H&E stain
10X Mag.

Appendix 1

Indirect *in situ* RT-PCR for lysyl oxidase. Case 10843A3; DCIS/IDC/Ductal hyperplasia.

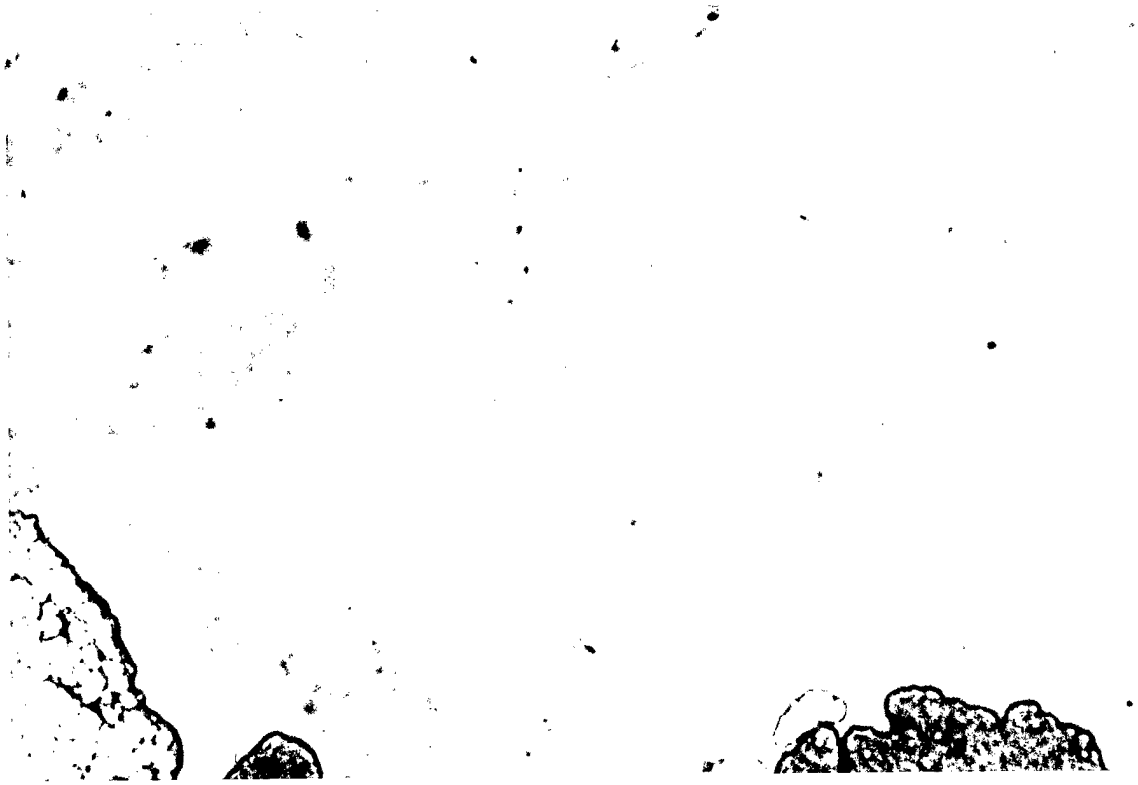


FIG. 11
DCIS/IDC
10X Mag.

FIG. 12
Normal ducts in non-cancerous area of tissue
10X Mag.

Appendix 1

Indirect *in situ* RT-PCR for lysyl oxidase. Case 10843A3; DCIS/IDC/Ductal hyperplasia.

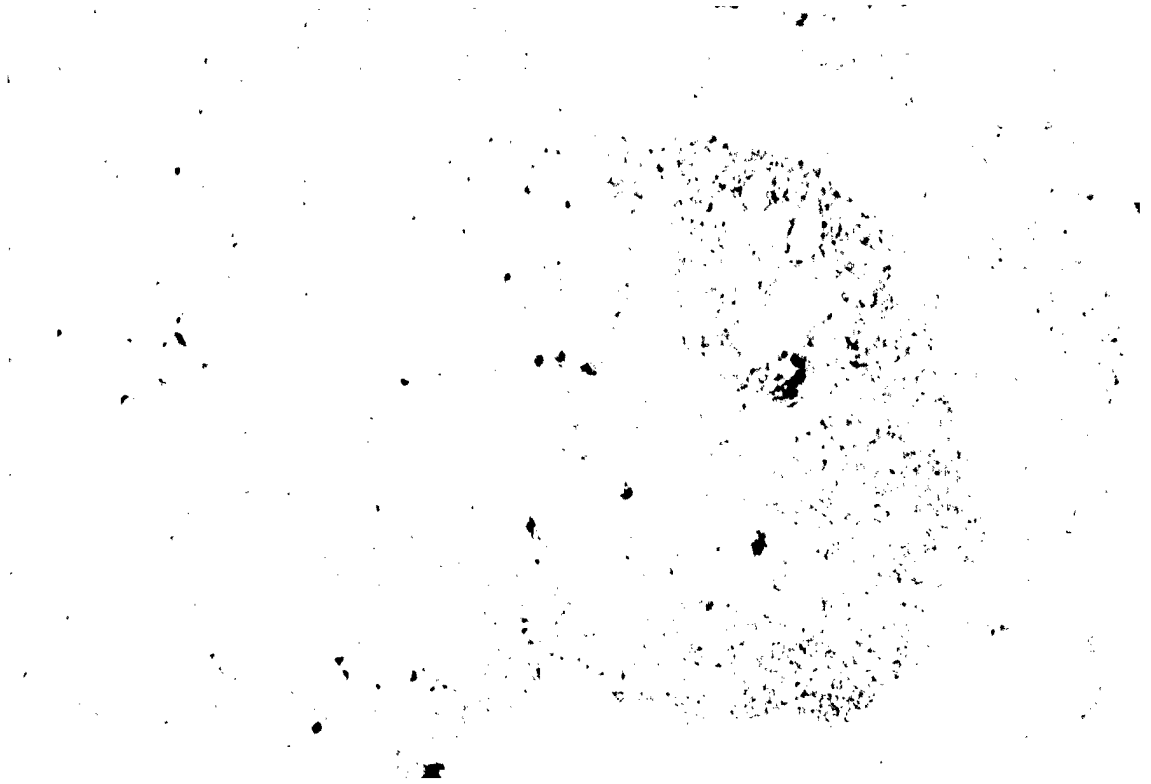


FIG. 13
DCIS
25X Mag.

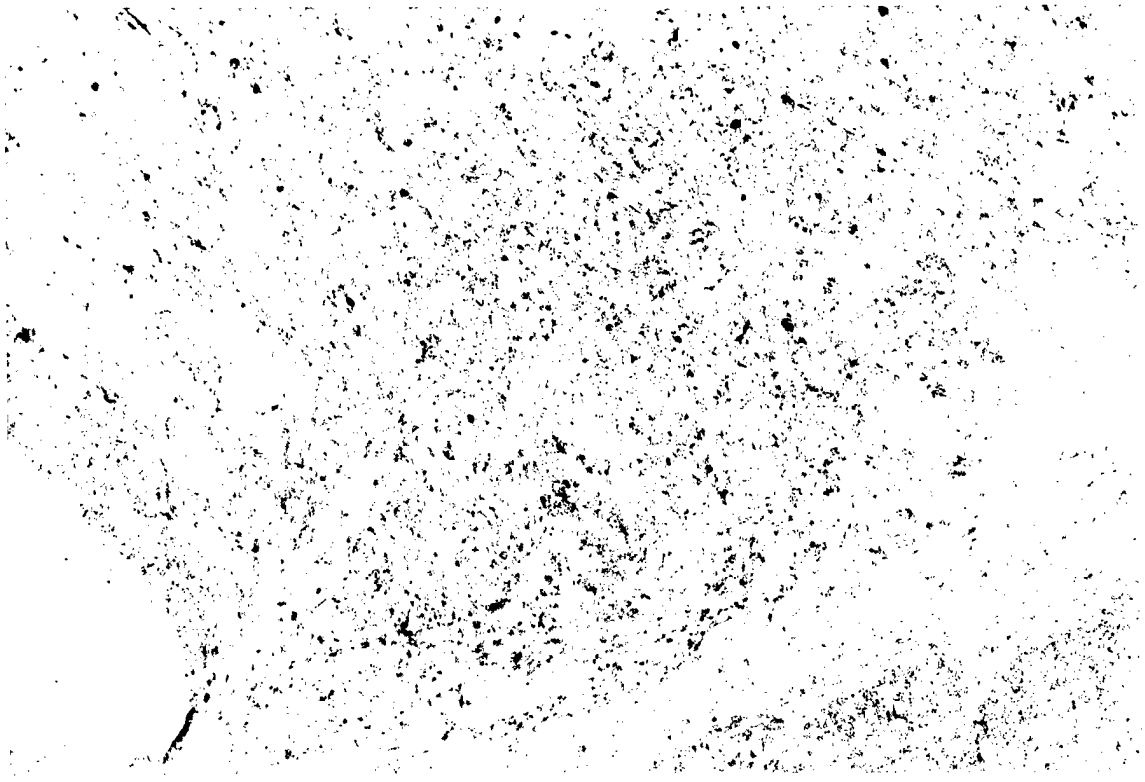


FIG. 14
IDC
25X Mag.

Appendix 1

Indirect *in situ* RT-PCR for *ras*. Case 10843A3; DCIS/IDC/Ductal hyperplasia.

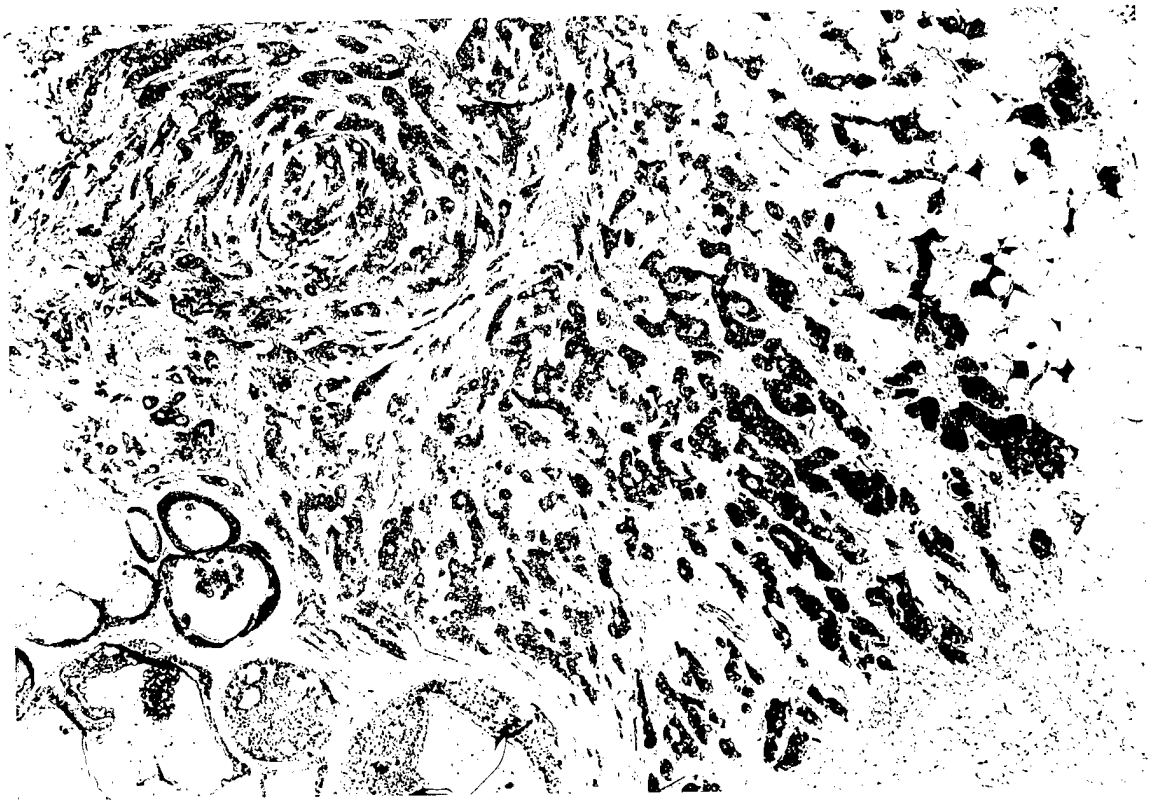


FIG. 15
DCIS/IDC
10X Mag.

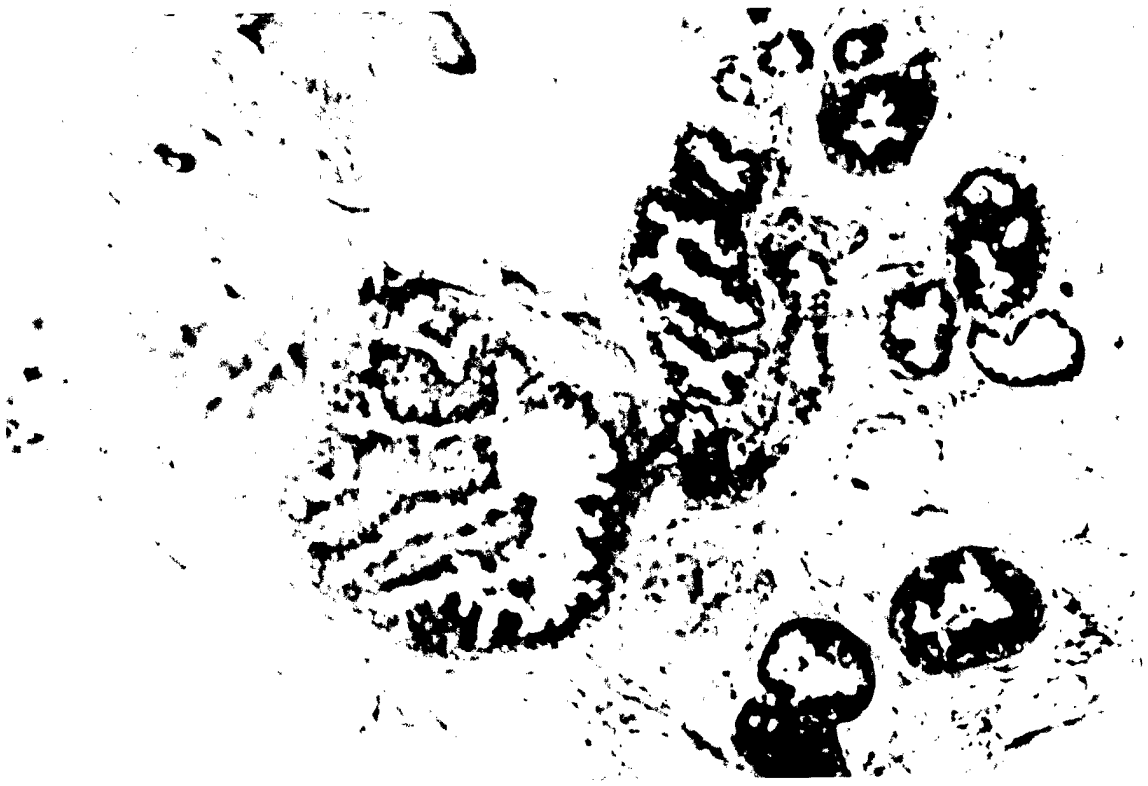


FIG. 16
DCIS
25X Mag.

Appendix 1

Negative control (lacking RT and Taq). Case 10843A3; DCIS/IDC/Ductal hyperplasia.

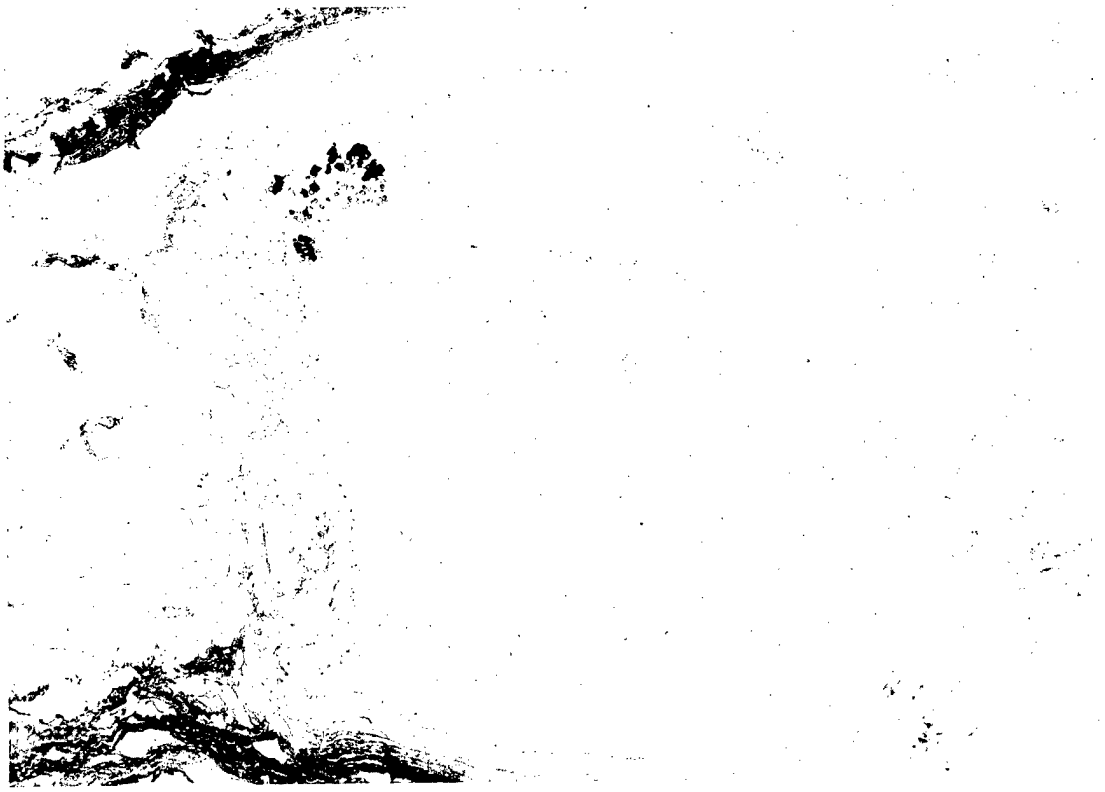


FIG. 17
10X Mag.

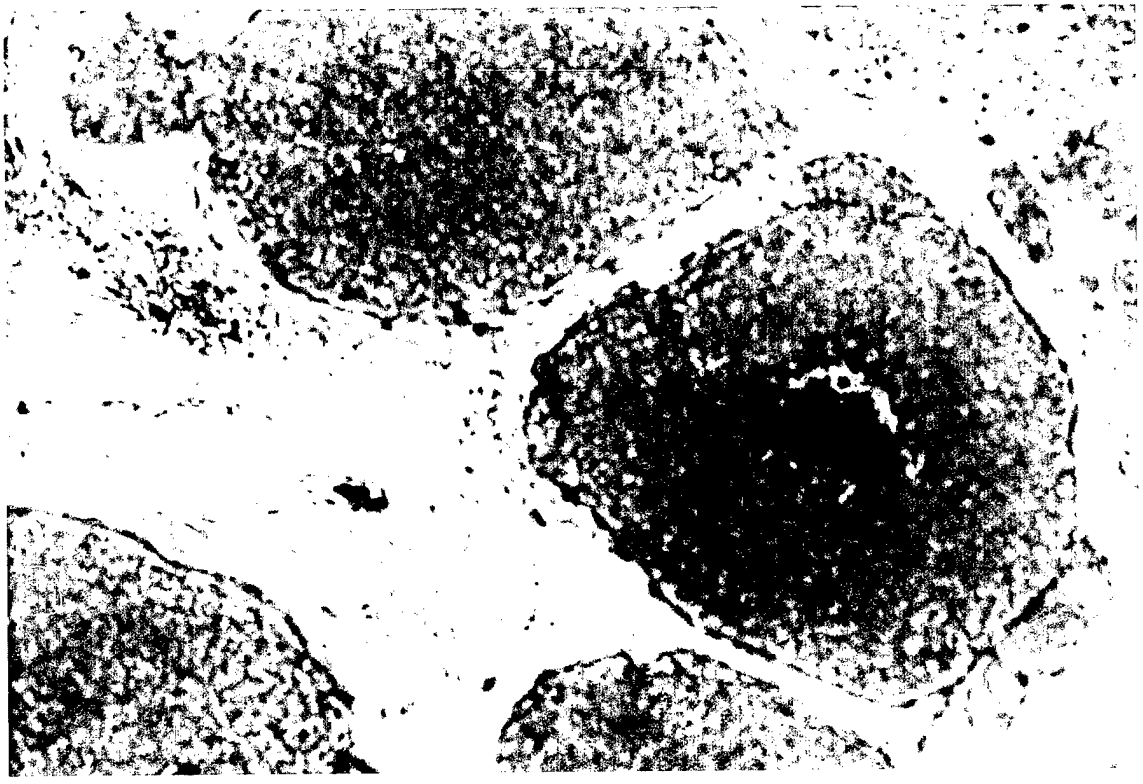
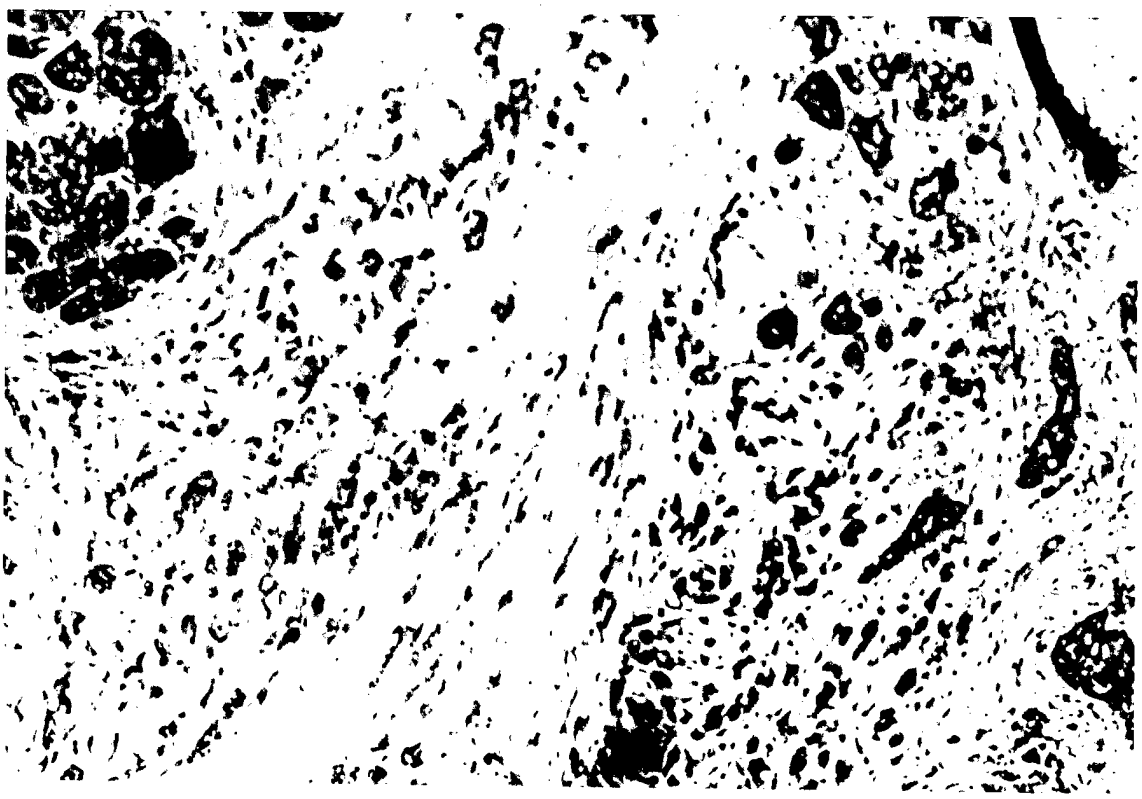


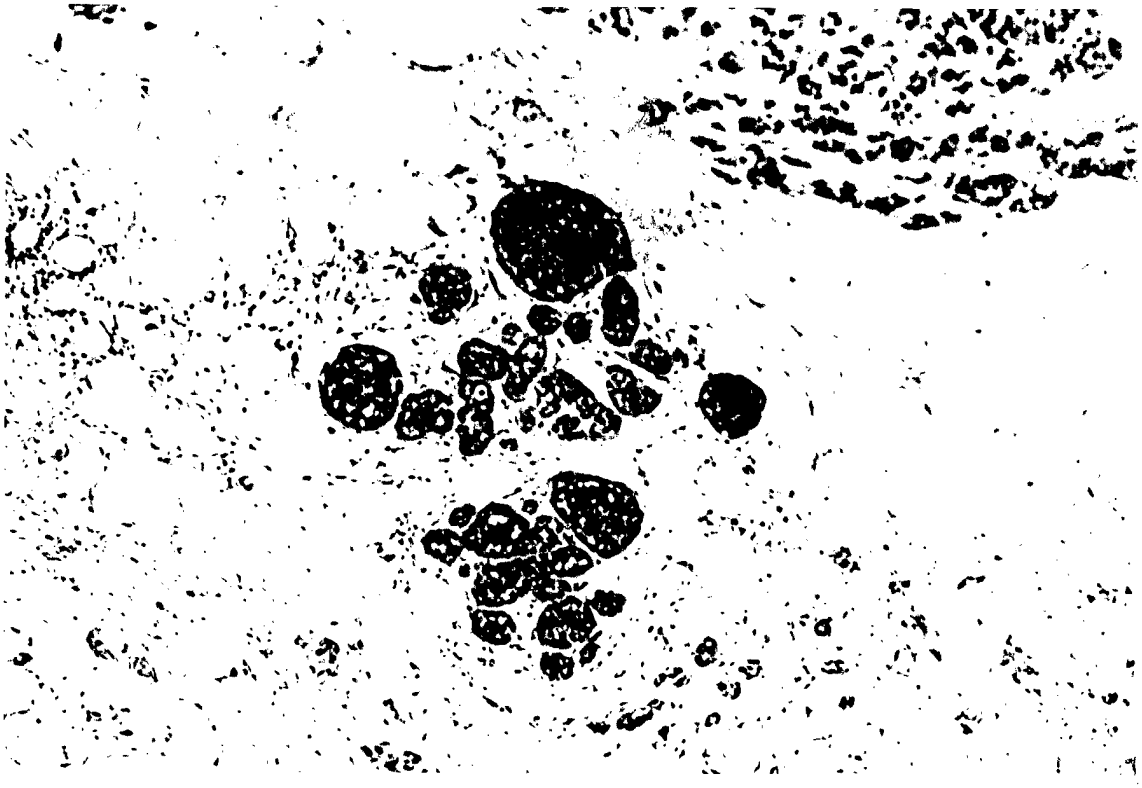
FIG. 18
H&E stain
DCIS
25X Mag.

Appendix 1

Indirect *in situ* RT-PCR for lysyl oxidase. Case 94SP1015 Mixed ILC/LCIS/IDC/DCIS



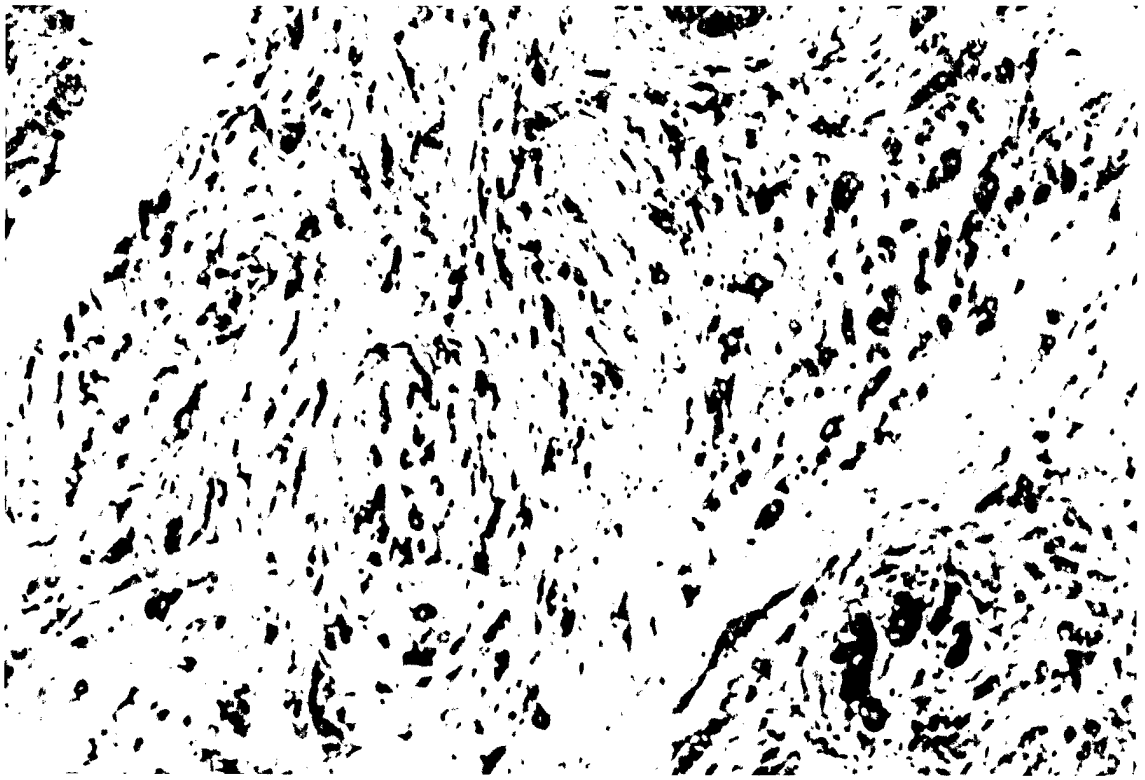
ILC/LCIS
FIG. 19
25X Mag.



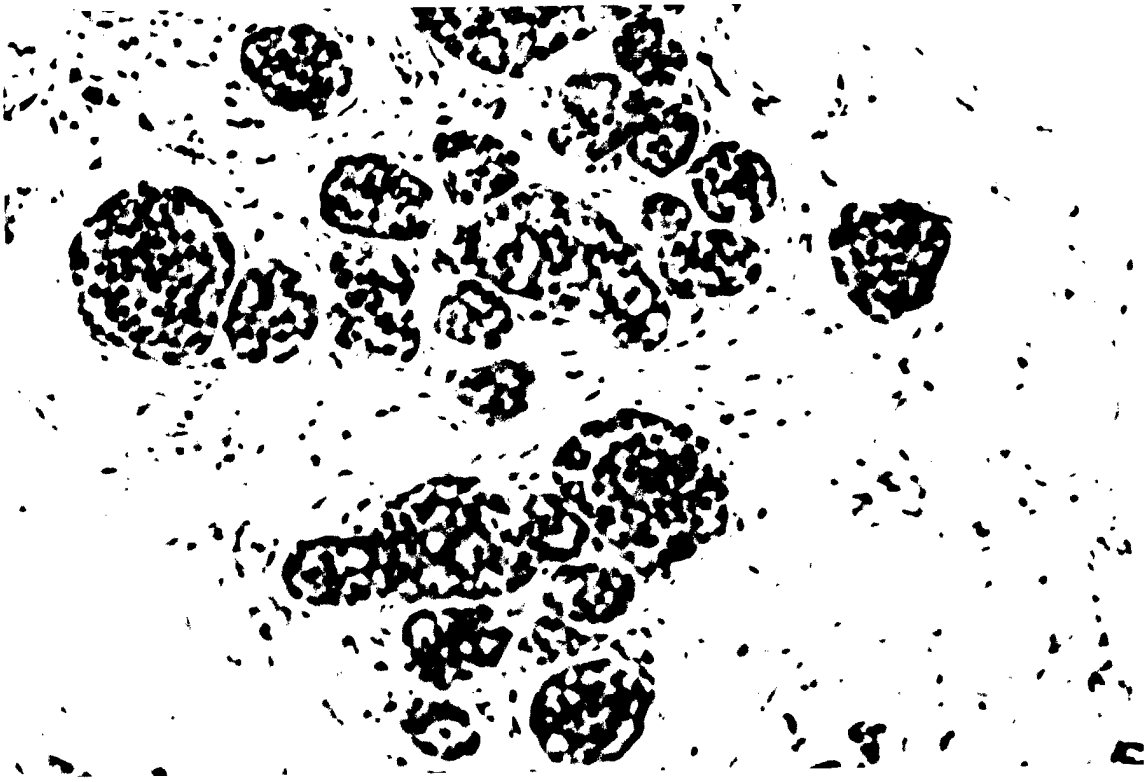
LCIS
FIG. 20
25X Mag.

Appendix 1

Indirect *in situ* RT-PCR for *ras*. Case 94SP1015 Mixed ILC/LCIS/IDC/DCIS



ILC
FIG. 21
25X Mag.



LCIS
FIG. 22
50X Mag.

Appendix 1

Negative control (lacking RT and Taq). Case 94SP1015 Mixed ILC/LCIS/IDC/DCIS.

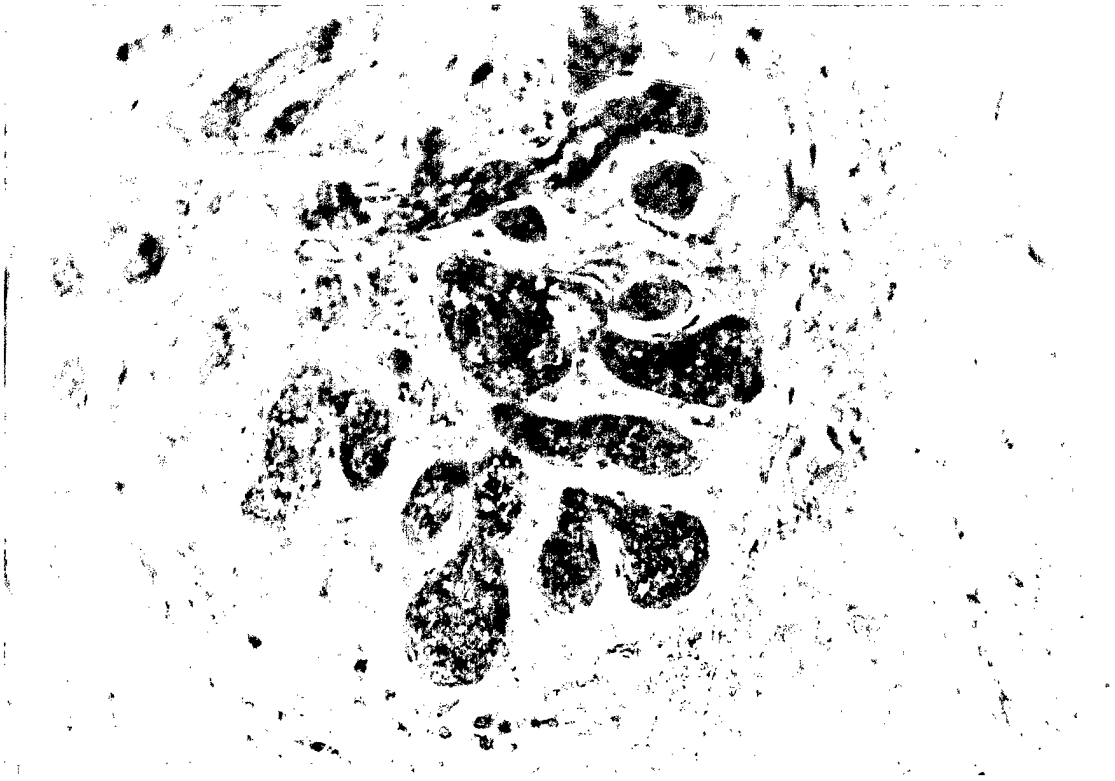


FIG. 23
50X Mag.

Appendix 1

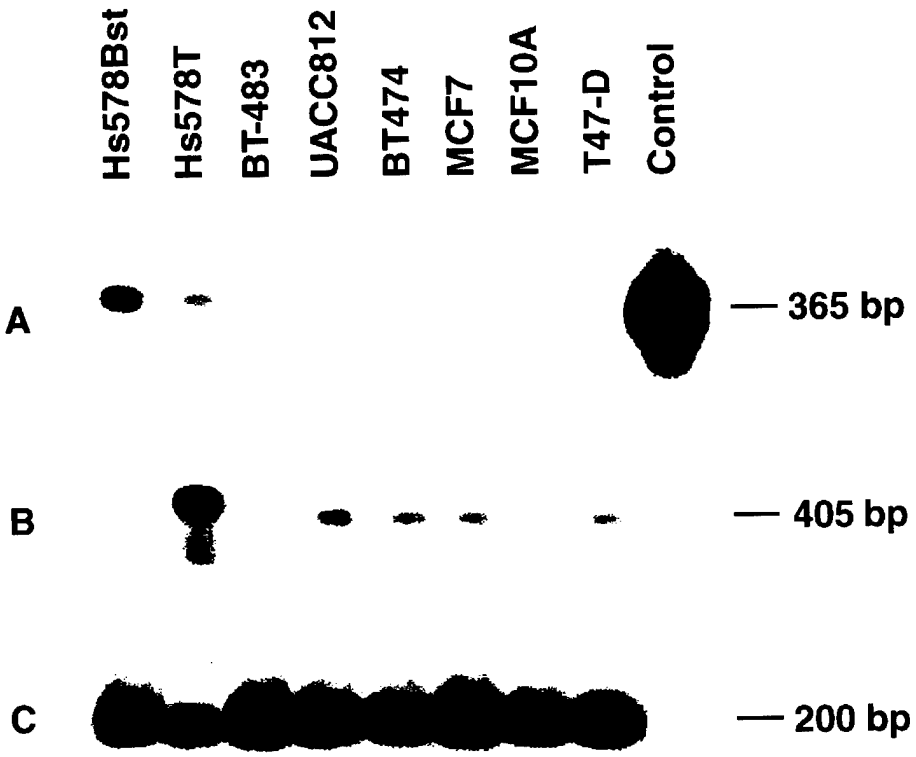


Fig. 24. RT-PCR products were generated with gene-specific primer sets, using human breast cancer line total cell RNA. Products were electrophoresed on 2.5% agarose gels, transferred to a nylon membrane, and hybridized with radiolabeled probe for: A. Lysyl oxidase, B. *ras*, C. G3PDH. The control lane contained PCR product for lysyl oxidase from a cloned cDNA.